FEBS Letters 586 (2012) 766–771

FEBS Letters

journal homepage: www.FEBSLetters.org

HBXIP upregulates CD46, CD55 and CD59 through ERK1/2/NF-κB signaling to protect breast cancer cells from complement attack

Wenjing Cui [a,1], Yu Zhao [a,1], Changliang Shan [b], Guangyao Kong [b], Nan Hu [a], Yiwen Zhang [a], Shuai Zhang [b], Weiying Zhang [b], Yingyi Zhang [a], Xiaodong Zhang [b,*], Lihong Ye [a,*]

[a] Department of Biochemistry, College of Life Sciences, State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin 300071, China
[b] Department of Cancer Research, Institute for Molecular Biology, College of Life Sciences, Nankai University, Tianjin 300071, China

ARTICLE INFO

Article history:
Received 16 December 2011
Revised 18 January 2012
Accepted 19 January 2012
Available online 27 January 2012

Edited by Masayuki Miyasaka

Keywords:
HBXIP
mCRP
CDC
ERK1/2
NF-κB

ABSTRACT

Hepatitis B X-interacting protein (HBXIP) is able to enhance migration of breast cancer cells. However, the role of HBXIP in regulation of complement-dependent cytotoxicity (CDC) in breast cancer is not understood. Here, we report that HBXIP contributes to protecting breast cancer cells from CDC by upregulating membrane-bound complement regulatory protein (mCRPs), including CD46, CD55 and CD59. We found that HBXIP upregulated mCRPs through activating p-ERK1/2/NF-κB. Interestingly, the knockdown of CD59 was able to block the HBXIP-enhanced breast tumor growth in animal. Thus, we conclude that HBXIP upregulates CD46, CD55 and CD59 through p-ERK1/2/NF-κB signaling to protect breast cancer from CDC.

1. Introduction

Mammalian hepatitis B X-interacting protein (HBXIP) is a conserved 18 kDa protein that was originally identified through its interaction with hepatitis B virus X protein [1]. We previously found that positive rates of HBXIP in primary tumor tissues and metastatic lymph tissues were 75% (38/49) and 94% (36/38), respectively [2]. However, HBXIP expression was not detectable in normal breast tissues (0/11), suggesting that HBXIP is a novel oncoprotein. Normal cells are protected from inappropriate complement attack by membrane-bound complement regulatory proteins (mCRPs), including CD46, CD55 and CD59, which prevent complement activation or block the formation of the terminal cytolytic membrane attack complex (MAC) [3]. It has been reported that tumor cells also express mCRPs, which serve as an effective mechanism to evade immune surveillance and complement-dependent cytotoxicity (CDC) [4–6]. Increased expression of mCRPs has been identified in several different malignancies, including colorectal, gastric, lung, renal, hepatoma and breast cancers [7–9]. However, the mechanism by which complement regulatory proteins are regulated in tumor cells during CDC remains unclear.

In the present study, we report that HBXIP is able to regulate CD46, CD55 and CD59 through p-ERK1/2/NF-κB signaling to protect breast cells from complement attack. The finding provides new insight into the mechanism of HBXIP in regulation of CDC.

2. Materials and methods

2.1. Cell culture and treatment

MCF-7 and LM-MCF-7 cells were cultured in RPMI 1640 medium (Gibco, USA) [10]. The breast cancer cell line MDA-MB-231 were cultured in DMEM medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, 100 U/ml streptomycin and 1% glutamine streptomycin in 5% CO_2 at 37 °C. Cells were plated into 6-well plates. Twenty-four hours later, the cells were placed in serum-free medium for an additional 12 h. In brief, MCF-7 and MDA-MB-231 cells containing overexpressed or silenced HBXIP, were treated with 30 μM PD98059 (an inhibitor of MEK1, Sigma, USA) for 1–4 h or 60 μM PDTC (an inhibitor of NF-κB, Sigma) for 1, 2 h, respectively.

Abbreviations: HBXIP, hepatitis B X-interacting protein; mCRPs, membrane-bound complement regulatory proteins; CDC, complement-dependent cytotoxicity; NF-κB, nuclear factor κB; IκB, inhibitor κB; ERK, extracellular regulatory kinase; JNK, c-Jun N-terminal kinase

* Corresponding authors. Fax: +86 22 23501385.
E-mail addresses: zhangxd@nankai.edu.cn (X. Zhang), yelihong@nankai.edu.cn (L. Ye).

[1] These authors contributed equally to this work.

0014-5793/$36.00 Crown Copyright © 2012 Published by Elsevier B.V. on behalf of Federation of European Biochemical society. All rights reserved.
doi:10.1016/j.febslet.2012.01.039

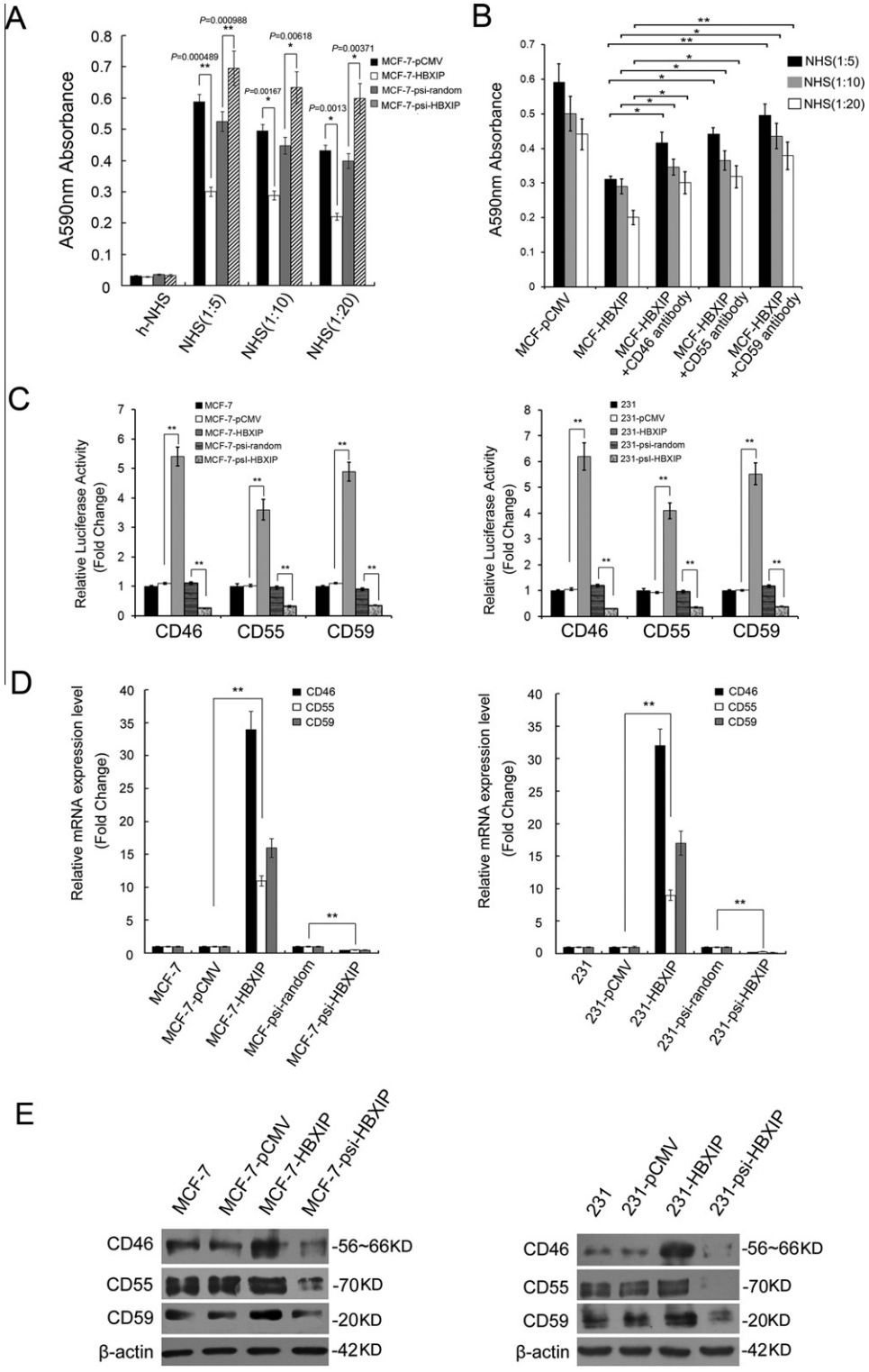

Fig. 1. HBXIP decreases the sensitivity of breast cancer cells to CDC via upregulating mCRPs. (A) The effect of HBXIP on the response of MCF-7 cells to CDC was examined by Trypan blue absorbance assay in stable HBXIP (or HBXIP RNAi)-transfection system. The heated-normal human serum (h-NHS) was served as negative control. (B) The 25 ng/ml antibodies of mCRPs, such as rabbit anti-CD46, rabbit anti-CD55 or rabbit anti-CD59 were added into 2 ml medium of MCF-7-pCMV cells or MCF-7-HBXIP cells for 30 min, respectively. Then, the effect of HBXIP on the response of MCF-7 cells to CDC was examined by trypan blue absorbance assay in the cells. (C) The effect of HBXIP-overexpression on activation of CD46, CD55 and CD59 was examined by luciferase reporter gene assay in MCF-7 or MDA-MB-231 (231) cells. $^{**}P < 0.001$ *vs* control, Student's *t*-test. (D and E) The expression levels of mCRPs were examined by qRT-PCR and western blot analysis in breast cell lines, respectively.

2.2. Complement-mediated cytolysis (CDC) assay

The extent of complement-mediated tumor cell death was determined by Trypan blue absorbance assay, according to the method as previously described [11].

2.3. Tumor formation in nude mice

The tumorigenicity of MCF-7-pCMV stable cells (pretreated with psi-random), MCF-7-HBXIP stable cells (pretreated with psi-random) [12] or MCF-7-HBXIP stable cells (pretreated with

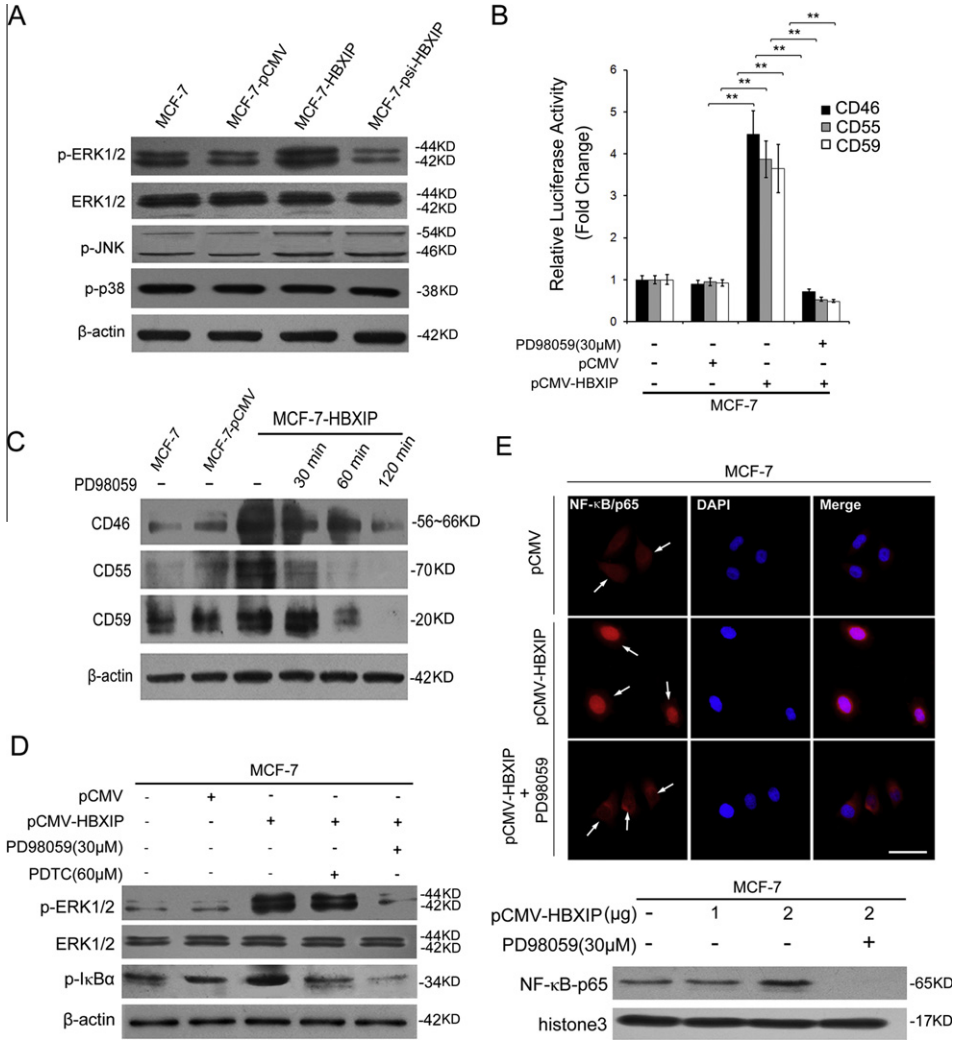

Fig. 2. HBXIP activates NF-κB signaling through increasing p-ERK1/2. (A) The phosphate levels of p-ERK1/2, p-JNK, or p-p38 were tested by western blot analysis in MCF-7-HBXIP cells relative to MCF-7 or MCF-7-pCMV cells (B) Promoter activities of CD46, CD55 and CD59 was examined by luciferase reporter gene assay in transiently HBXIP-transfected MCF-7 cells after treatment with 30 μM PD98059 for 120 min. $^{**}P < 0.001$, *vs* control, Student's *t*-test. (C) The protein expression levels of mCRPs were determined by western blot in MCF-7-HBXIP cells after treatment with 30 μM PD98059 in time course. (D) The phosphate level of p-IκBα was test with the transfection of HBXIP and a treatment of 60 μM PDTC by western blot analysis. (E) NF-κB-p65 nuclear translocation mediated by HBXIP was examined with 30 μM PD98059 by immunofluorescence staining and nuclear western blot in MCF-7 cells. Bar means 50 μm.

pSilencer-CD59) was measured, respectively. As follows, aliquots (0.2 ml) with 2×10^6 cells were injected subcutaneously into 4-week-old nude mice ($n = 6$ each group). All studies were approved by the Animal Care Committee of Nankai University.

2.4. Statistical analysis

Statistical analysis was performed using Sigma Plot 2001 (Systat Software Inc., Richmond, CA). Statistical significance was assessed by comparing mean values (±S.D.) using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.001$). $P < 0.05$ was considered as significant.

3. Results

3.1. HBXIP decreases the sensitivity of breast cancer cells to CDC via upregulating mCRPs

Recently, we reported that HBXIP was involved in breast cancer cell migration [2]. We observed the effect of HBXIP on CDC in breast cancer cells because HBXIP is an important oncoprotein. In this study, our data showed that HBXIP decreased the sensitivity of the cells to CDC in MCF-7 cells (Fig. 1A), suggesting that HBXIP is involved in CDC. Fig. 1B showed that the blocking of mCRPs using the antibodies of CD46, CD55 and CD59, respectively, was able to rescue the HBXIP-induced sensitivity of the cells to CDC in MCF-7 cells, supporting that CD46, CD55 and CD59 were involved in the HBXIP-induced CDC. To further explore the relationship between HBXIP and mCRPs, we generated several stable cell lines with overexpression or knockdown of HBXIP, called MCF-7-HBXIP (overexpression of HBXIP), MCF-7-psi-HBXIP (knockdown of HBXIP), MDA-MB-231-HBXIP (overexpression of HBXIP) and MDA-MB-231-psi-HBXIP (knockdown of HBXIP), respectively. Our results showed that the relative luciferase activities of CD46, CD55 and CD59 were significantly higher in HBXIP-transfected MCF-7 and MDA-MB-231 cells than that in control cells ($^{*}P < 0.05$, $^{**}P < 0.001$ *vs* MCF-7 or MDA-MB-231, Student's *t*-test) (Fig. 1C). Furthermore, western blot and qRT-PCR analysis showed that the overexpression or knockdown of HBXIP was able to regulate the expression of CD46, CD55 and CD59 in MCF-7 and MDA-MB-231 (Fig. 1D, E, S1A and S1B). All above data strongly suggest that HBXIP is able to upregulate the expression of CD46, CD55 and CD59.

3.2. HBXIP upregulates mCRPs via activating NF-κB signaling involving increasing p-ERK1/2

It has been reported that ERK contributes to CDC resistance in a human immortalized myelogenous leukemia line [13] and the CDC resistance of breast cancer cells is associated with aberrant activation of MAPK [14]. Thus, we supposed that MAPK may be involved in the HBXIP-mediated upregulation of mCRPs. Interestingly, we found that the level of p-ERK1/2, but not p-JNK or p-p38, was increased in MCF-7-HBXIP cells (Fig. 2A). Our data showed that the treatment with 30 μM PD98059 (an inhibitor of MEK) abolished the increased promoter activities and protein expression of CD46, CD55 and CD59 mediated by HBXIP in MCF-7 cells ($^{**}P < 0.001$, *vs* control, Student's *t*-test, Fig. 2B and C). The above data suggest that HBXIP is able to upregulate mCRPs through activating ERK1/2. Moreover, we found that PDTC (an inhibitor of NF-κB) failed to abolish the increased level of p-ERK1/2 mediated by overexpressing HBXIP in MCF-7 cells. However, PD98059 was able to abolish the increased level of p-IκBα in above cells (Fig. 2D). Immuno-fluorescence staining revealed that the treatment with 30 μM PD98059 attenuated the nuclear translocation of the NF-κB-p65 subunit in transient-HBXIP transfected MCF-7 cells. Similarly, we also found that the similar results in the nucleus proteins by western blot analysis (Fig. 2E). Therefore, these data show that the activation of NF-κB mediated by HBXIP is in a p-ERK1/2 signaling-dependent manner in breast cancer cells.

To validate the further mechanism of upregulation of CD46, CD55 and CD59, we examined the effect of NF-κB on these upregulations. Luciferase reporter gene assay showed that the treatment with PDTC abolished the increased promoter activities of CD46, CD55 and CD59 when HBXIP was overexpressed by transient transfection with pCMV-HBXIP plasmid in MCF-7 and MDA-MB-231 cells ($^{*}P < 0.05$; $^{**}P < 0.001$, *vs* controls, Student's *t*-test, Fig. 3A). Western blot analysis showed that PDTC was able to abolish the upregulation of CD46, CD55 and CD59 in MCF-7-HBXIP and MDA-MB-231-HBXIP cells in a time-dependent manner (Fig. 3B). In addition, the NF-κB-p65 siRNA resulted in the downregulation of mCRPs in MCF-7-HBXIP cells and MDA-MB-231-HBXIP cells (Fig. 3C). These data suggest that NF-κB signaling pathway is responsible for the upregulation of CD46, CD55 and CD59 mediated by HBXIP in breast cancer cells.

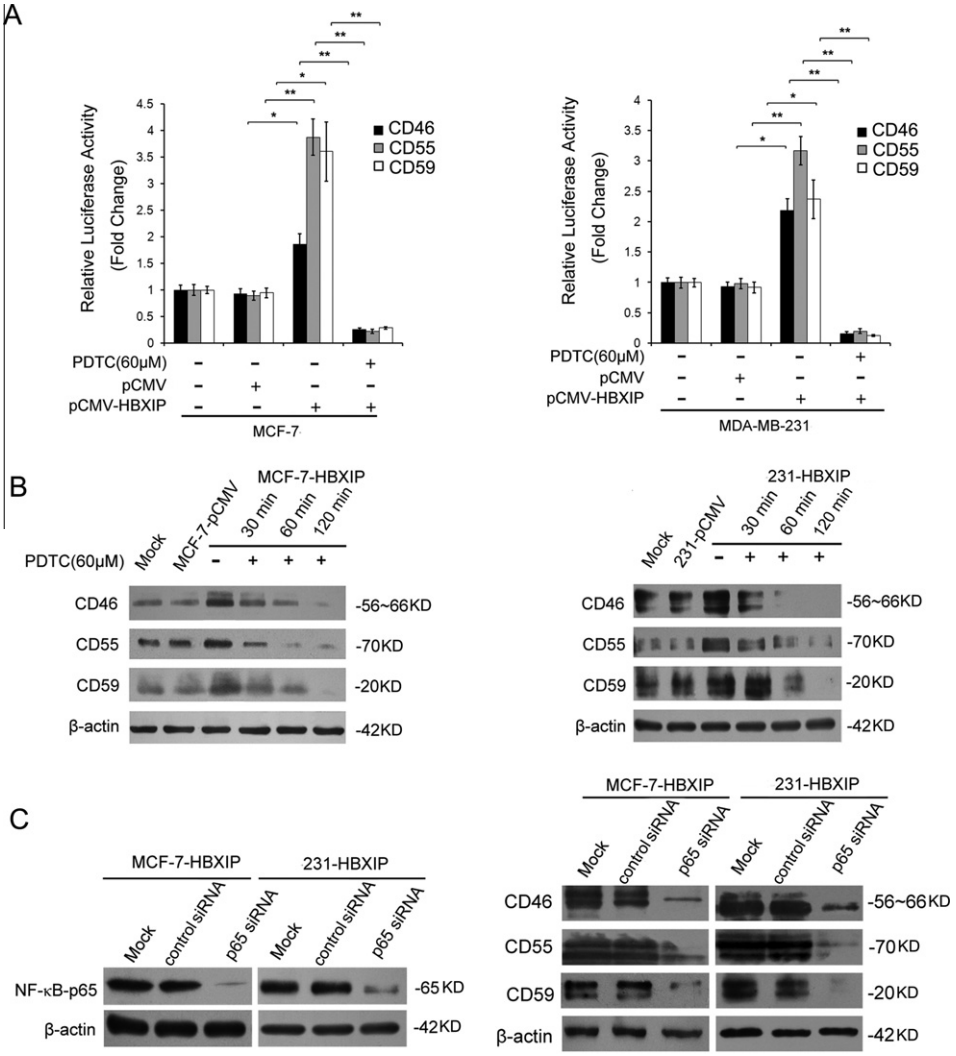

Fig. 3. The activation NF-κB is responsible for the upregulation of CD46, CD55 and CD59. (A) The effect of NF-κB on enhancement of promoter activities of mCRPs mediated by HBXIP was measured by luciferase reporter gene assay in transiently HBXIP-transfected MCF-7 cells and MDA-MB-231 (231) cells after treatment with 60 μM PDTC for 120 min. $^{*}P < 0.05$; $^{**}P < 0.001$, *vs.* controls, Student's *t*-test. (B) Expression levels of CD46, CD55 and CD59 were detected by western blot analysis in MCF-7-HBXIP and MDA-MB-231-HBXIP (231-HBXIP) cells after treatment with 60 μM PDTC in time course. (C) The expression level of NF-κB-p65 was measured by western blot analysis. MCF-7-HBXIP and MDA-MB-231-HBXIP (231-HBXIP) cells were transfected by NF-κB-p65 siRNA.

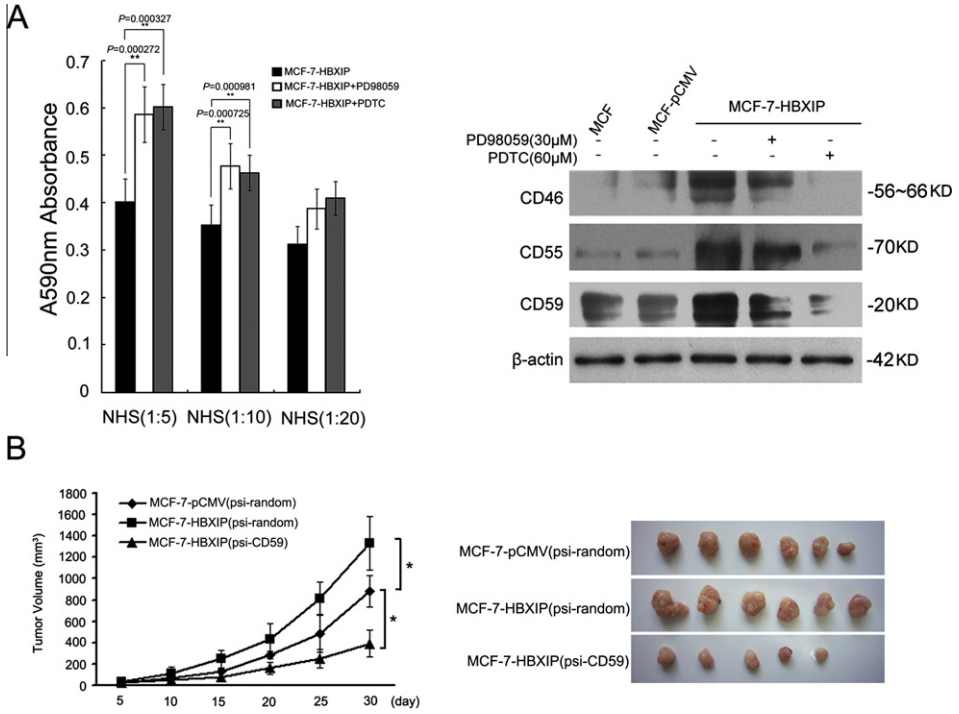

Fig. 4. p-ERK1/2 and NF-κB are involved in CDC mediated by HBXIP. (A) The effect of p-ERK1/2 or NF-κB on response of MCF-7-HBXIP cells to CDC was examined by trypan blue absorbance assay in different dilutions of normal human serum after treatment with 30 μM PD98059 or 60 μM PDTC. Meanwhile, the downregulation of CD46, CD55 and CD59 were examined by western blot analysis. $^{**}P < 0.001$, Student's *t*-test. (B) Tumor size was measured every 5 days using a vernier caliper. Each bar shows the mean ± S.D. ($n = 6$) (left panel). The tumors from xenograft mouse models were shown (right panel). $^{*}P < 0.05$, Student's *t*-test.

3.3. p-ERK1/2 and NF-κB are involved in CDC mediated by HBXIP

In function, we found that the inhibition of ERK1/2 and NF-κB was able to sensitize the MCF-7-HBXIP cells to CDC (Fig. 4A) ($^{**}P < 0.001$, *vs* control, Student's *t*-test). We then examined the effect of CD59 on tumor growth in HBXIP-overexpression MCF-7 breast cancer cells in mice using psi-CD59. Interestingly, the suppression of tumor growth was observed in nude mice injected by MCF-7-HBXIP cells pretreated with psi-CD59 ($^{*}P < 0.05$, Student's *t*-test, Fig. 4B), suggesting that HBXIP enhances cell growth in mice is in a CD59-dependent manner. The knockdown efficiency of CD59 by psi-CD59 was detected by western blot in MCF-7 cell (Fig. S1C). Thus, we conclude that HBXIP contributes to the protection of breast cancer cells from CDC through upregulation of mCRPs involving a cascade signal of p-ERK1/2/ NF-κB.

4. Discussion

Recently, we have reported that HBXIP is involved in the proliferation and migration of breast cancer cells [2]. In this study, we investigated the role of HBXIP in regulation of mCRPs in breast cancer cells. We found that HBXIP was inversely associated with the sensitivity of the cells to CDC in MCF-7 cells (Fig. 1). mCRPs play an important role in the immune evasion strategy of tumors by inhibiting complement cascade activation [5]. Interestingly, HBXIP expression is positively correlated with the expression of mCRPs in breast cancer cells. Then, we further demonstrated that HBXIP was able to upregulate the expression of CD46, CD55 and CD59 at the levels of promoter activity, mRNA expression and protein expression in breast cancer cells.

Next, we focused on the investigation of mechanism of upregulation of mCRPs mediated by HBXIP. According to the report that the survival capability of breast cancer cells is associated with aberrant activation of MAPK [14] and ERK contributes to CDC resistance in a human immortalized myelogenous leukemia line [13], we supposed that MAPK may be involved in the HBXIP-mediated upregulation of mCRPs. Then, we showed that HBXIP increased the phosphorylation of ERK1/2, but not p-JNK or p-p38, in MCF-7-HBXIP cells. Indeed, it was confirmed that the increase of p-ERK1/2 level was responsible for upregulating mCRPs (Fig. 2). Because we previously reported that HBXIP activates NF-κB in hepatoma cells [12], the relationship between p-ERK1/2 and NF-κB in HBXIP-mediated upregulation of mCRPs was required to be identified. Interestingly, we found that the activation of NF-κB mediated by HBXIP is in a p-ERK1/2 signaling-dependent manner in breast cancer cells. These data are consistent with a report showing that NF-κB is a downstream effector of p-ERK1/2 [15]. We further demonstrated that NF-κB was involved in HBXIP-mediated upregulation of mCRPs in breast cancer cells.

Consequently, increased complement resistance conferred by these mCRPs has been proposed as a mechanism that facilitates tumor survival or survival of metastasizing tumor cells in the circulation [15–17]. According to our above observations, we need to show all the factors involving the upregulation of mCRPs, such as p-ERK1/2, NF-κB and CD59, are responsible for CDC in breast cancer cells. Our finding suggests that the activation of ERK1/2 and NF-κB plays a critical role in regulation of mCRPs involving CDC, which is consistent with the previous reports [13,18].

Taken together, we summarize a model that HBXIP is a novel regulator for CD46, CD55 and CD59, involving ERK1/2/NF-κB signaling, which contributes to protecting the cells from complement attack (Fig. S2). Our data provide new insights into the mechanism of regulation of mCRPs in breast cancer cells.

Acknowledgments

This work was supported in part by Natural Scientific Foundation of China (No. 81071623), National Basic Research Program of China (973 Program, No. 2011CB512113, No. 2009CB521702).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.039.

References

[1] Melegari, M., Scaglioni, P.P. and Wands, J.R. (1998) Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. J. Virol. 72, 1737–1743.

[2] Hu, N., Zhang, J., Cui, W., Kong, G., Zhang, S., Yue, L., Bai, X., Zhang, Z., Zhang, W., Zhang, X. and Ye, L. (2011) MiR-520b regulates migration of breast cancer cells by targeting hepatitis B X-interacting protein and interleukin-8. J. Biol. Chem. 286, 13714–13722.

[3] Maio, M., Brasoveanu, L.I., Coral, S., Sigalotti, L., Lamaj, E., Gasparollo, A., Visintin, A., Altomonte, M. and Fonsatti, E. (1998) Structure, distribution, and functional role of protectin (CD59) in complement-susceptibility and in immunotherapy of human malignancies (Review). Int. J. Oncol. 13, 305–318.

[4] Rooney, I.A., Heuser, J.E. and Atkinson, J.P. (1996) GPI-anchored complement regulatory proteins in seminal plasma. An analysis of their physical condition and the mechanisms of their binding to exogenous cells. J. Clin. Invest. 97, 1675–1686.

[5] Gorter, A. and Meri, S. (1999) Immune evasion of tumor cells using membrane-bound complement regulatory proteins. Immunol. Today 20, 576–582.

[6] Fishelson, Z., Donin, N., Zell, S., Schultz, S. and Kirschfink, M. (2003) Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol. Immunol. 40, 109–123.

[7] Durrant, L.G., Chapman, M.A., Buckley, D.J., Spendlove, I., Robins, R.A. and Armitage, N.C. (2003) Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients. Cancer Immunol. Immunother. 52, 638–642.

[8] Inoue, T., Yamakawa, M. and Takahashi, T. (2002) Expression of complement regulating factors in gastric cancer cells. Mol. Pathol. 55, 193–199.

[9] Spiller, O.B., Criado-Garcia, O., Rodriguez De Cordoba, S. and Morgan, B.P. (2000) Cytokine-mediated up-regulation of CD55 and CD59 protects human hepatoma cells from complement attack. Clin. Exp. Immunol. 121, 234–241.

[10] You, J., Mi, D., Zhou, X., Qiao, L., Zhang, H., Zhang, X. and Ye, L. (2009) A positive feedback between activated extracellularly regulated kinase and cyclooxygenase/lipoxygenase maintains proliferation and migration of breast cancer cells. Endocrinology 150, 1607–1617.

[11] Uliasz, T.F. and Hewett, S.J. (2000) A microtiter trypan blue absorbance assay for the quantitative determination of excitotoxic neuronal injury in cell culture. J. Neurosci. Methods 100, 157–163.

[12] Wang, F.Z., Sha, L., Zhang, W.Y., Wu, L.Y., Qiao, L., Li, N., Zhang, X.D. and Ye, L.H. (2007) Involvement of hepatitis B X-interacting protein (HBXIP) in proliferation regulation of cells. Acta Pharmacol. Sin. 28, 431–438.

[13] Kraus, S., Seger, R. and Fishelson, Z. (2001) Involvement of the ERK mitogen-activated protein kinase in cell resistance to complement-mediated lysis. Clin. Exp. Immunol. 123, 366–374.

[14] Dey, A., Wong, E., Kua, N., Teo, H.L., Tergaonkar, V. and Lane, D. (2008) Hexamethylene bisacetamide (HMBA) simultaneously targets AKT and MAPK pathway and represses NF kappaB activity: implications for cancer therapy. Cell Cycle 7, 3759–3767.

[15] Chen, B.C., Yu, C.C., Lei, H.C., Chang, M.S., Hsu, M.J., Huang, C.L., Chen, M.C., Sheu, J.R., Chen, T.F., Chen, T.L., Inoue, H. and Lin, C.H. (2004) Bradykinin B2 receptor mediates NF-kappaB activation and cyclooxygenase-2 expression via the Ras/Raf-1/ERK pathway in human airway epithelial cells. J. Immunol. 173, 5219–5228.

[16] Varsano, S., Rashkovsky, L., Shapiro, H., Ophir, D. and Mark-Bentankur, T. (1998) Human lung cancer cell lines express cell membrane complement inhibitory proteins and are extremely resistant to complement-mediated lysis; a comparison with normal human respiratory epithelium in vitro, and an insight into mechanism(s) of resistance. Clin. Exp. Immunol. 113, 173–182.

[17] Rushmere, N.K., Knowlden, J.M., Gee, J.M., Harper, M.E., Robertson, J.F., Morgan, B.P. and Nicholson, R.I. (2004) Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer. Int. J. Cancer 108, 930–936.

[18] Chen, K.H., Weng, M.S. and Lin, J.K. (2007) Tangeretin suppresses IL-1beta-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. Biochem. Pharmacol. 73, 215–227.